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PRINCIPAL INVESTIGATOR: Margot P. Cleary, Ph.D.

CONTRACTING ORGANIZATION: University of Minnesota
                                                        Minneapolis, MN 55455-2070

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Calorie restriction has been reported to protect rodents from many different cancers. With respect to prostate cancer, a protective effect of energy restriction on development of spontaneous prostate tumors in Lobund-Wistar rats and tumors developing from transplanted prostate tumor tissue or cells in mice and rats has been published. However we have found that in female rodents intermittent caloric restriction is more protective than chronic restriction in preventing transgenic mammary tumor development. Here, we determined how intermittent versus chronic calorie restricted affected development of prostate cancer in transgenic TRAMP mice. A 25% reduction in caloric intake was utilized. Intermittent-restricted mice had significant delay in the age of tumor detection and age at death compared to ad libitum and chronic restricted mice. Serum leptin to adiponectin ratio was lower following intermittent restriction and may indicate an environment that inhibits cell proliferation. In tumor and genital-urinary tissue we are attempting to identify metabolic pathways to target for prevention and/treatment strategies. In particular we are assessing aspects of the IGF-I, adiponectin and leptin axes. The results of this study provide further evidence that the manner in which calories are consumed has a significant impact of development of some malignancies.

15. SUBJECT TERMS
prostate cancer, TRAMP, transgenic mice, caloric restriction

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INTRODUCTION:

A number of prospective epidemiological studies indicate that as body weight and/or energy intake increase so does the risk for prostate cancer. In rodent studies chronic calorie restriction is associated with extended life expectancy and decreased incidence of many malignancies. Due to a lack of suitable animal models of prostate cancer, only a few studies have addressed issues of nutrition intervention in the progression of this disease. However, results of these studies support a protective effect of energy restriction on spontaneous prostate tumor development in Lobund-Wistar rats [1;2] and on transplanted tumor/cell prostate tumor growth in mice and rats [3], although a mechanism of action has not been identified. There are limitations to the application of these models to the human disease process. Recent introduction of the TRAMP (transgenic adenocarcinoma mouse prostate) mouse provides a model that shares many characteristics with human prostate cancer [4;5], but use of these mice in nutritional studies has been limited. In the present study we used TRAMP mice to evaluate their response to chronic calorie restriction, as well as to intermittent caloric restriction/refeeding. These studies are based on our recent report that these two interventions resulted in decreased incidence and extended latency of oncogene-induced mammary tumors in MMTV-TGF-α female mice [6]. Furthermore, we found that the intermittent caloric restriction/refeeding regimen was more protective than chronic restriction in preventing mammary tumorigenesis. Here TRAMP mice were followed to determine their response to these interventions with respect to age of prostate cancer detection and metastases rates. Serum and tissue samples have been obtained to determine the role of the insulin-like growth factor (IGF) axis in the protective action of caloric restriction. Additionally based on the increasing interest in adipose tissue derived proteins, adipokines, as growth factors for different types of malignancies and the fact that serum levels of these, in particular leptin and adiponectin, are affected by calorie intake and body weight we have expanded our studies to include them.

BODY:
Progress in relation to Revised Statement of Work 2/13/03 (attached)

TASK 1 & 2. Establish breeding colony & set up genotyping assay.
MONTH 0-3. Order 6 male TRAMP mice (maximum number that could be ordered at one time) and 25 nontransgenic female mice for breeding. Set up breeding. Set up genotyping assay and genotype mice produced. Rebreed mice to expand breeding colony.

Progress: Now completed with respect to breeding regimens and genotyping.

TASK 3. Breed mice for EXPERIMENT 1A- LONGITUDINAL STUDY.
MONTHS 4-6. Breed mice to produce one third to one half of mice needed for this study. If one estimates 8 pups per litter, 1 out of 4 pups will be TRAMP males = two TRAMP males per litter. We will need a total of 160 TRAMP males = 80 litters. Genotype offspring. Assign mice to experimental groups. Set up immunohistochemistry assays.

Progress: Breeding completed.

TASK 4. Complete enrollment of mice for EXPERIMENT 1A and 1B SERIAL STUDY.
MONTHS 7-12. Continue breeding to compete EXPERIMENT 1A. Three to four rounds of breeding will probably be needed to supply enough mice. Genotype mice as they are produced. Assign mice to experimental groups. Once longitudinal study is complete begin assigning mice to serial study for EXPERIMENT 1B.

Progress: Breeding completed.

TASK 5. Follow mice in EXPERIMENT 1A and 1B.
MONTHS 6-21. Monitor food intake, body weight and prostate tumor development in TRAMP mice. When age, tumor size and/or animal condition dictates euthanize mice and perform autopsies. Euthanize nontransgenic age-matched mice to correspond to those TRAMP mice with tumors. Euthanize mice that reach terminal ages of 48 or 50 wk of age. Record results and when study complete do statistical analyses of results.

Progress: All mice have been euthanized. All pathology reports have been summarized analyzed to determine tumor and metastases status. A large amount of data has been obtained and statistical analyses has been done to determine what samples to analyze further. Serum analyses were done to determine IGF-I levels as well as leptin, insulin, adiponectin and IL-6. Here we will highlight what we have obtained to date.

Food Intake. By prevention of overeating during the refeeding period we maintained an overall degree of restriction of ~25% for the intermittent-restricted and chronic-restricted (pair-fed) groups. This makes interpretation of the results more straight forward as we previously found with female mice that some of them overate relative to the ad libitum fed mice during refeeding resulting in overall caloric restriction being in the range of 10-

20% although this was still highly protective [6;7]. Specifics of the food intake data were presented in last year's progress report.
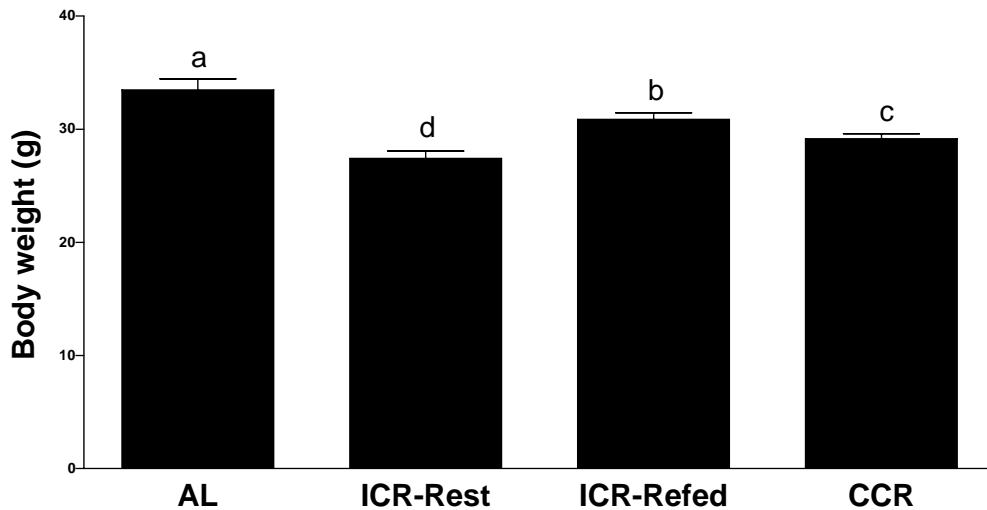

PROGRESS LONGITUDINAL STUDY

Body weight. The body weight curves for the mice in the Longitudinal Study are shown in Figure 1. In contrast to our earlier study using female TGF-α mice the intermittent restricted mice did not regain weight during refeeding periods and "catch up" to the body weight attained by ad libitum-fed mice. However, as indicated above in this protocol we restrained food intake during refeeding periods so that mice did not "overshoot" the intake of the ad libitum fed mice. The ad libitum fed mice exhibited a slow steady increase in body weight until about 40 weeks of age and then a plateau was reached. The chronic restricted mice had a moderate weight gain at the beginning of the intervention and then their body weights were maintained. As expected from the experimental design, the intermittent restricted mice lost weight during restriction periods and regained body weight with refeeding. Note that the last body weight recorded on the body weight graph represents the one week of refeeding as mice were euthanized during the 50th week.
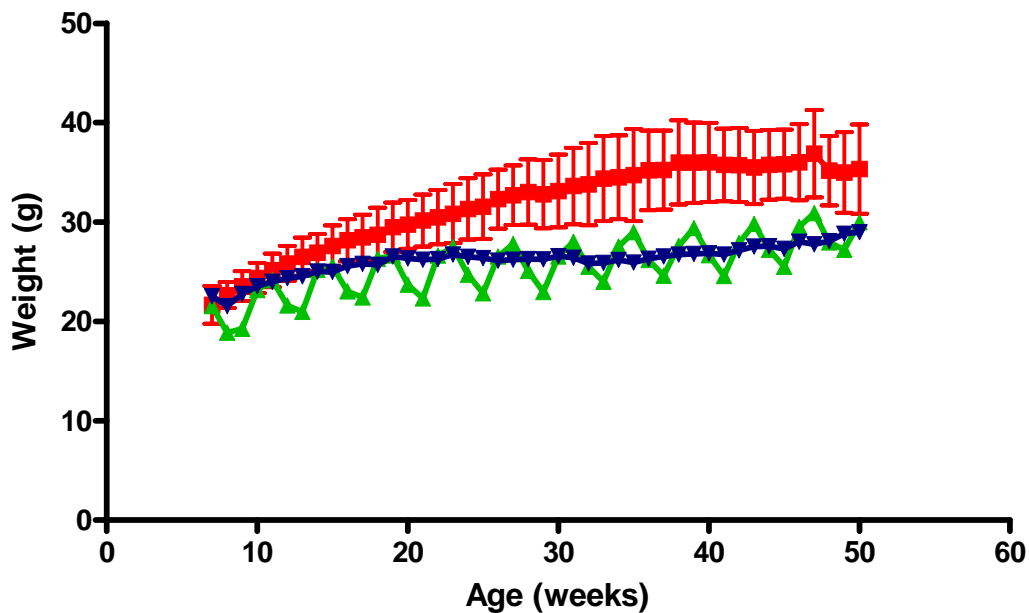
Figure 1: Body weight curves for TRAMP mice in longitudinal study. Ad libitum (■) N=4-41 depending upon age; Intermittent-Restricted (▲), N=22-101 dependent upon age; Chronic-Restricted (▼) N=21-79 dependent upon age. For clarity error bars not included for the two restricted groups. ANOVA P<0.0001, Ad libitum groups are significantly different from each restricted group at P<0.001 while there was no difference between the two restricted groups.

Final body weights for mice in the longitudinal study are presented in Figure 2. The intermittent restricted mice are separated by euthanization at 48 and 50 weeks of age. It can be seen that the ad libitum mice weighed more than all other groups at death while intermittent restricted mice euthanized during refeeding periods had significantly higher body weights than both the intermittent restricted-restricted and the chronic restricted groups.

Figure 2. Final body weights for TRAMP mice in Longitudinal Study. **AL = Ad libitum-fed mice   (N = 39); ICR-Rest = Intermittent Calorie-Restricted euthanized during a restriction period (N = 41); ICR-Refed = Intermittent Calorie-Restricted euthanized during a refeeding period (n = 55); CCR = Chronic Calorie-Restricted (N = 75). ANOVA P<0.0001; columns with different superscripts significantly different from each other.**

Tumor Detection and Mortality: As presented in Figure 3, age of tumor detection was significantly delayed in the intermittent restricted mice in comparison to both the ad libitum and chronic restricted pair-fed mice. In contrast there were no differences between ad libitum fed and chronic restricted mice.

TRAMP Mice AC detection by any route
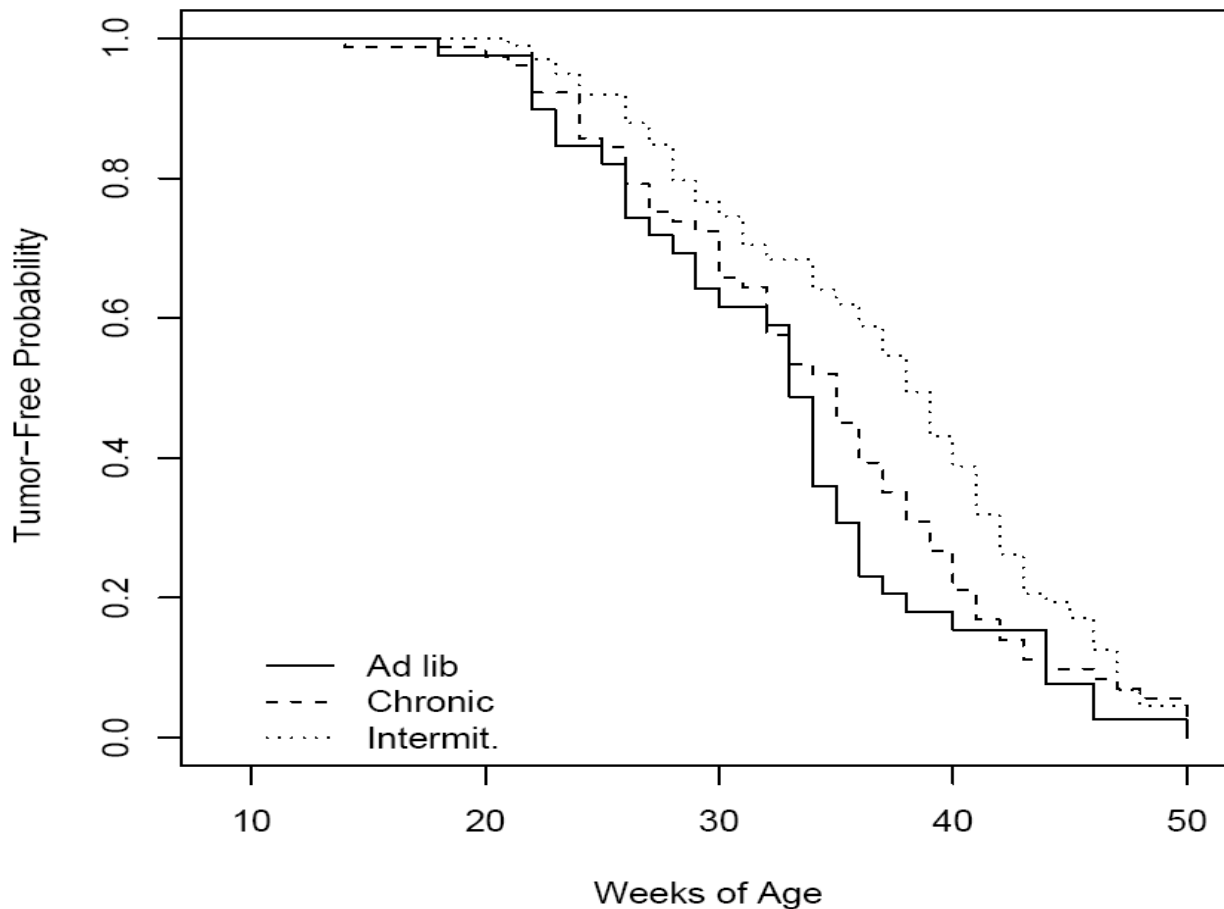
Figure 3. Age of tumor detection by Kaplan-Meier plot. Chi-square test for all mice had a test statistic of 8.301 on 2 degrees of freedom (P=0.016). Comparison for ad libitum-fed and chronic restricted mice is not significant. Intermittent restricted mice had significantly delayed age of tumor detection compared to ad libitum (P<0.007) mice and chronic restricted (P=0.042) mice.

Additionally, age at death (Figure 4) exhibited a shift to the right indicating significantly older age at death for the intermittent restricted mice. Results are presented as Kaplan Meier Plots with Chi Square Analysis. There were no differences between the ad libitum and chronic restricted mice with respect to age of death.

TRAMP Mice All Cause Mortality
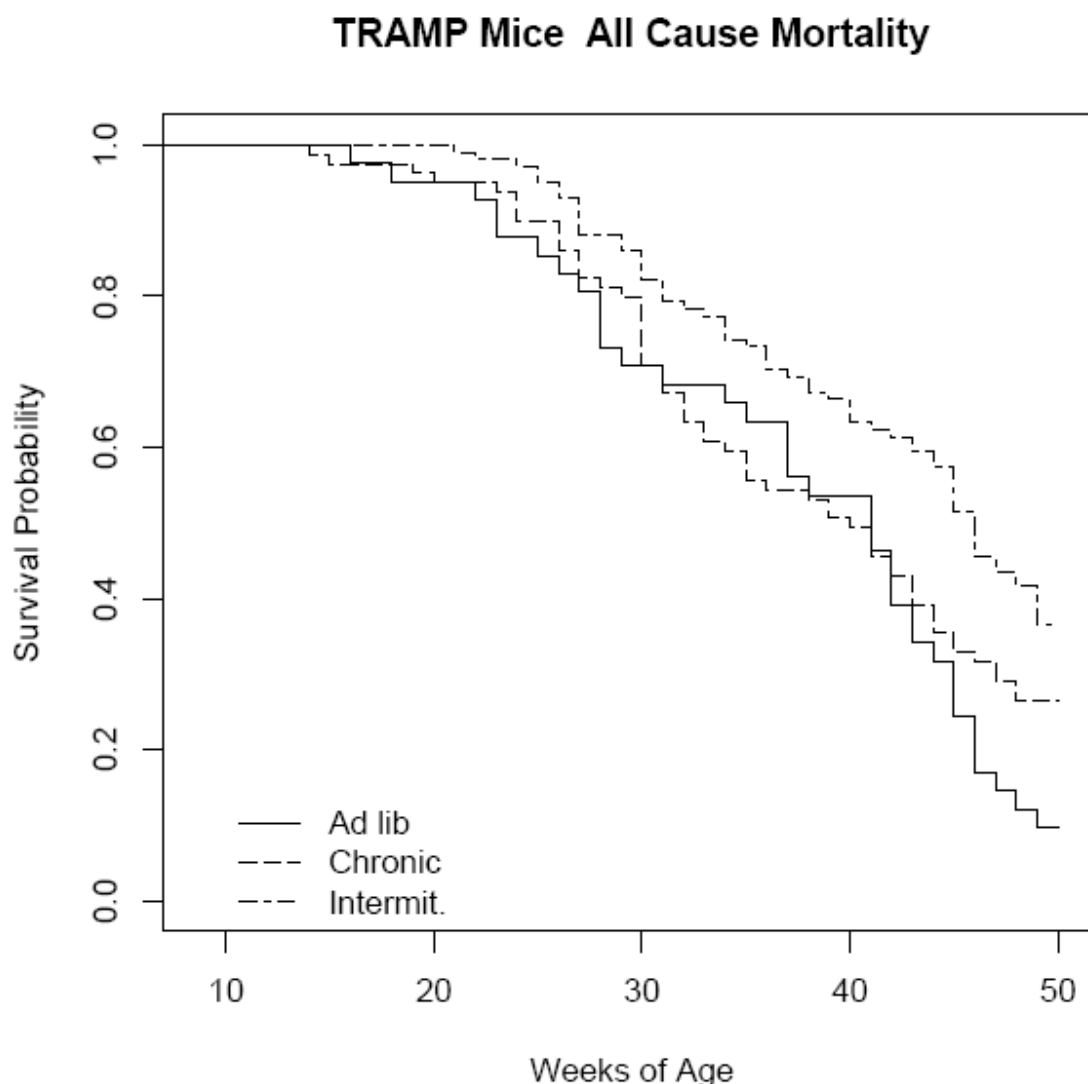
Figure 4. Kaplan-Meier survival curve for TRAMP mice in the Longitudinal Study. Mortality among the three groups had a Chi-square test statistic of 12.498 on 2 degrees of freedom and was statistically significant at P=0.009. There was no significant difference between the ad libitum fed and chronic restricted groups. Intermittent restricted mice lived longer in comparison to both ad libitum (P=0.0004) and chronic restricted (P=0.0265) mice.

We also examined the number of mice from each group that survived until designated terminal end point. Significantly more (39/101) intermittent restricted mice, 39%, reached this goal compared to only 21/79, 27%, of chronic restricted and 4/41, 10%, of the ad libitum fed mice (Chi Square analysis, P=0.027)

Tumor Grade and Metastasis: Pathological analyses of tumors and tissues have now been completed. As summarized in Table 1 there were no significant differences in the tumor grade among the three experimental groups. There was a slight increase in non-

adenocarcinoma tissue from the intermittent restricted mice and decrease in high grade tumors.

Table 1. Tumor Grade for TRAMP Mice in the Longitudinal Study.

Dietary group	Total enrolled	Non-adenocarcinoma	Low grade	Moderate grade	High grade
AL	41	4.9%	17.1%	41.5%	36.6%
CCR	79	5.1%	12.7%	43.0%	39.2%
ICR	101	8.9%	12.9%	46.5%	31.7%

Pearson's Chi-square = 2.65, 6 degrees of freedom, P=0.85.

In these TRAMP mice we identified metastases in the liver, lung, lymph nodes and kidney tissues. As shown in Table 2, there were no significant differences in percent mice with metastasis among the three dietary groups.

Table 2. Metastases Rates in TRAMP Mice in Longitudinal Study.

|	Number with Metastasis	Percent with Metastasis
AL	20/41	49%
ICR	47/101	47%
CCR	49/79	62%

P=0.5009 by Chi-square analysis.

Serum Analyses**:** In the original proposal we planned to measure IGF-I levels in the serum of the mice. As this study progressed the opportunity became available to measure additional serum factors in small volumes that may play a role in tumorigenesis. Luminex technology was used (University of Pittsburg, Cancer Center, Dr. Anna Loshkin) to measure leptin, adiponectin, and insulin. There has been particular interest in recent years in both leptin and adiponectin and their potential roles in prostate cancer [8-15]. Samples were obtained in the intermittent restricted mice that were euthanized at both the end of the final restriction period as well as the end of the final refeeding period. As outlined in Figure 5A there was little effect in IGF-I levels. Insulin, Figure 5B, tended to be higher in the intermittent restricted mice that were refed and the chronic restricted mice in comparison to the ad libitum fed mice while the intermittent restricted mice had an intermediate level. These findings may be more a reflection of the feeding schedule for the mice than a reflection of physiological response to long term diet intervention. Leptin levels (Figure 5C) were significantly reduced in the intermittent restricted-restricted mice in comparison to all

other groups while their adiponectin levels (Figure 5D) remained at the level of the other groups except for the chronic restricted mice. This results in a leptin to adiponectin ratio (Figure 5E) that was at least 50% lower in the intermittent restricted-restricted mice than the other groups. This is a very intriguing finding given the recent interest in adiponectin as a protective factor against cancer and warrants further investigation.
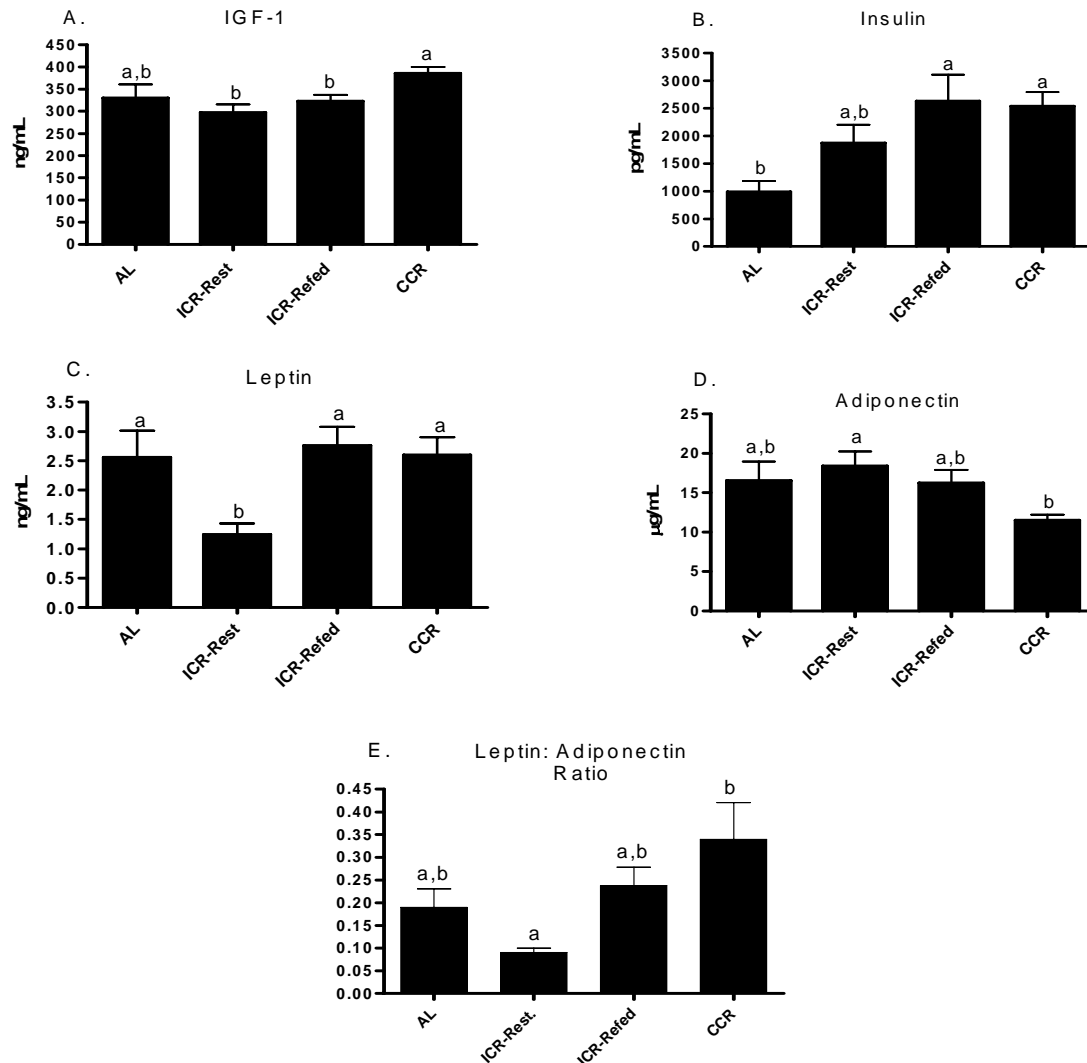
Figure 5. Serum analysis for TRAMP mice. A. IGF-1 AL (N=27) ICR-Rest (N=33), ICR-Refed (N=40), CCR (N=31). ANOVA P=0.0154. B. Insulin AL(N=35), ICR-Rest (N=38), ICR-Refed (N=41), CCR (N=66). ANOVA P=0.0030. C. Leptin, D. Adiponectin and E. Leptin: Adiponectin ratio AL (N=36), ICR-Rest (N=38), ICR-Refed (N=42), CCR (N=66). Leptin ANOVA P=0.0086. Adiponectin ANOVA P=0.0059. Leptin: Adiponectin ratio ANOVA P=0.0349. Columns with different superscripts significantly different from each other.

As shown in Figure 6 we also calculated the correlations of leptin and adiponectin to body weight and to fat pad weights. Leptin had a strong positive correlation with both factors (Figure 6A and 6C) while adiponectin was negatively correlated with body weight (Figure 6B) but not related to fat mass (Figure 6D).
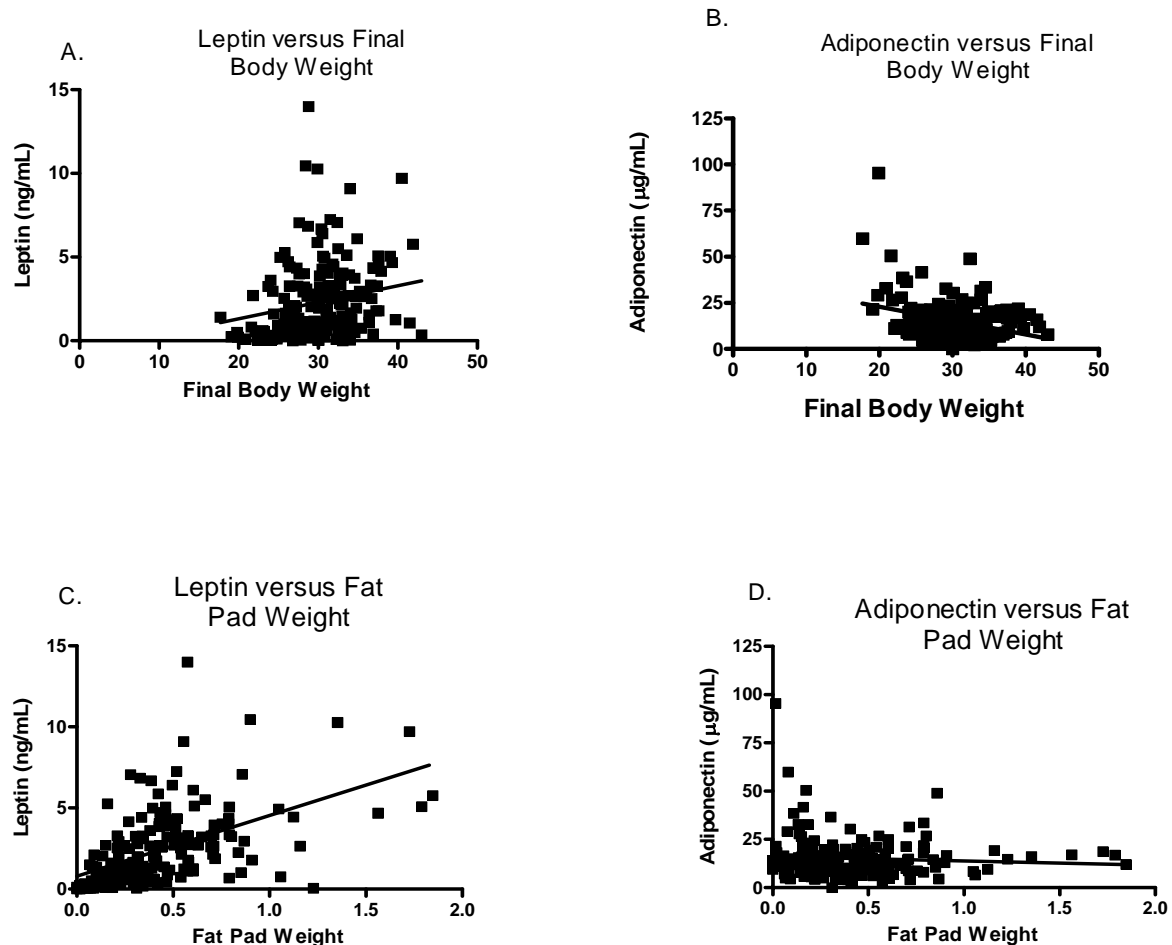
Figure 6. Correlations between serum leptin and adiponectin to final body weight and fat pad weight. For all graphs N=182. A. Leptin versus final body weight, r=0.2005, P=0.0066. B. Adiponectin versus final body weight, r= -0.3249, P<0.0001. C. Leptin versus fat pad weight, r=0.5394, P<0.0001. D. Adiponectin versus fat pad weight, r=-0.068, P=0.3597.

Body composition: Body composition was determined by DEXA in collaboration with Dr. Susan Perkins. Among TRAMP mice, there was no effect on bone growth for the mice in any groups when bone mineral content, area or bone mineral densities were determined (results not shown). Thus the intervention did not have a negative effect on bone

development. A comparison of body composition among the groups as well as to nonTRAMP mice was done and this is summarized in Table 3.

Table 3. Body Composition Results for TRAMP Mice in the Longitudinal Study.

Dietary Group	N	Bone Data			Tissue Data			
		BMD (g/cm^2)	BMC (g)	AREA (g/cm^2)	Lean (g)	Fat (g)	Total (g)	% Fat
AL TRAMP	24	0.05 ± 0.00	0.58 ± 0.09	10.97 ± 1.00	12.47 ± 2.56	6.68 ± 2.72	19.15 ± 4.73	34.05 ± 7.34
AL nonTRAMP	9	0.05 ± 0.00	0.64 ± 0.10	11.74 ± 1.06	14.16 ± 2.05	11.70 ± 4.25	25.88 ± 5.31	44.23 ± 8.30
IR TRAMP	70	0.05 ± 0.00	0.55 ± 0.08	10.55 ± 0.92	12.86 ± 3.16	5.19 ± 1.84	18.04 ± 3.96	28.78 ± 7.91
IR nonTRAMP	23	0.05 ± 0.00	0.52 ± 0.05	10.34 ± 0.84	13.96 ± 2.44	4.84 1.50	18.78 2.66	25.85 7.13
CR TRAMP	52	0.05 ± 0.00	0.53 ± 0.07	10.38 ± 0.87	14.23 ± 3.06	4.84 ± 1.78	19.08 ± 3.99	25.32 ± 7.20
CR nonTRAMP	20	0.05 ± 0.00	0.57 ± 0.06	10.35 ± 0.74	16.61 ± 2.50	6.87 ± 3.06	23.47 ± 3.69	28.62 ± 9.30

**AL = Ad libitum, IR = Intermittent-Restricted, CR = Chronic-Restricted.**

Soft tissue was not analyzed statistically due to concerns about tissue breakdown/ degradation that may have occurred during long term storage of carcasses.

A summary of some of the results for this aspect of the study are presented in Table 4. In general it can be seen that with respect to age of tumor detection, age at death and survival until study termination all are improved to a greater degree in the intermittent restricted mice compared to the chronic restricted mice. This is highlighted by the direct comparison of the two restricted groups receiving the same caloric and nutrient intakes differing only with the manner of consumption whereby the intermittent restricted mice have a delay in tumor detection and a delay in death. However metastases rates and tumor grade were not affected. It was also observed that the leptin to adiponectin ratio was reduced in the mice following two weeks of intermittent restriction and may reflect an environment that interferes with cell proliferation. This will be investigated in more detail in future studies.

Table 4. Highlights of Comparisons among TRAMP Mouse Groups in Longitudinal Study.

	Ad libitum vs Intermittent-Restricted	Ad libitum vs Chronic-Restricted	Intermittent-Restricted vs Chronic-Restricted
Food Intake	25% ↑	25% ↑	same
Tumor Number	same	same	same
Tumor Weight	same	same	same
Age at Tumor Palpation	14 % ↓	7 %↓	7 % ↑
Age at Death	10 % ↓	3 %↓	7 % ↑
Survival until Study Termination	75 % ↓	63 % ↓	48 % ↑
Metastasis Rate	same	same	same
Tumor Grade	same	same	same
Serum Leptin to Adiponectin Ratio	Higher by 100% following restriction	Lower by 100%	Lower 3 fold following restriction and 50% after refeeding

Tumor analyses: Based on preliminary studies in mammary tumors from intermittent restricted mice [7] protein determinations related to apoptosis and proliferation were performed on tumor tissue from tumor samples obtained from mice in the Longitudinal Study. As shown in Figure 7A there was a tendency for PCNA to be higher in intermittent refed and chronic restricted mice compared to the ad libitum fed and intermittent restricted mice but results were not significant. Caspase 3 (ns) levels (Figure 7B) tended to be elevated in all restricted groups as did Bcl-2 (Figure 7C, $P = 0.0351$). Cleaved PARP (Figure 7D) and Bax (Figure 7E) were variable and the Bax to Bcl-2 ratio (Figure 7F) was not significantly affected. We plan to measure adiponectin and leptin receptor levels in both normal and tumor tissues and will measure IGF-I receptor protein levels.
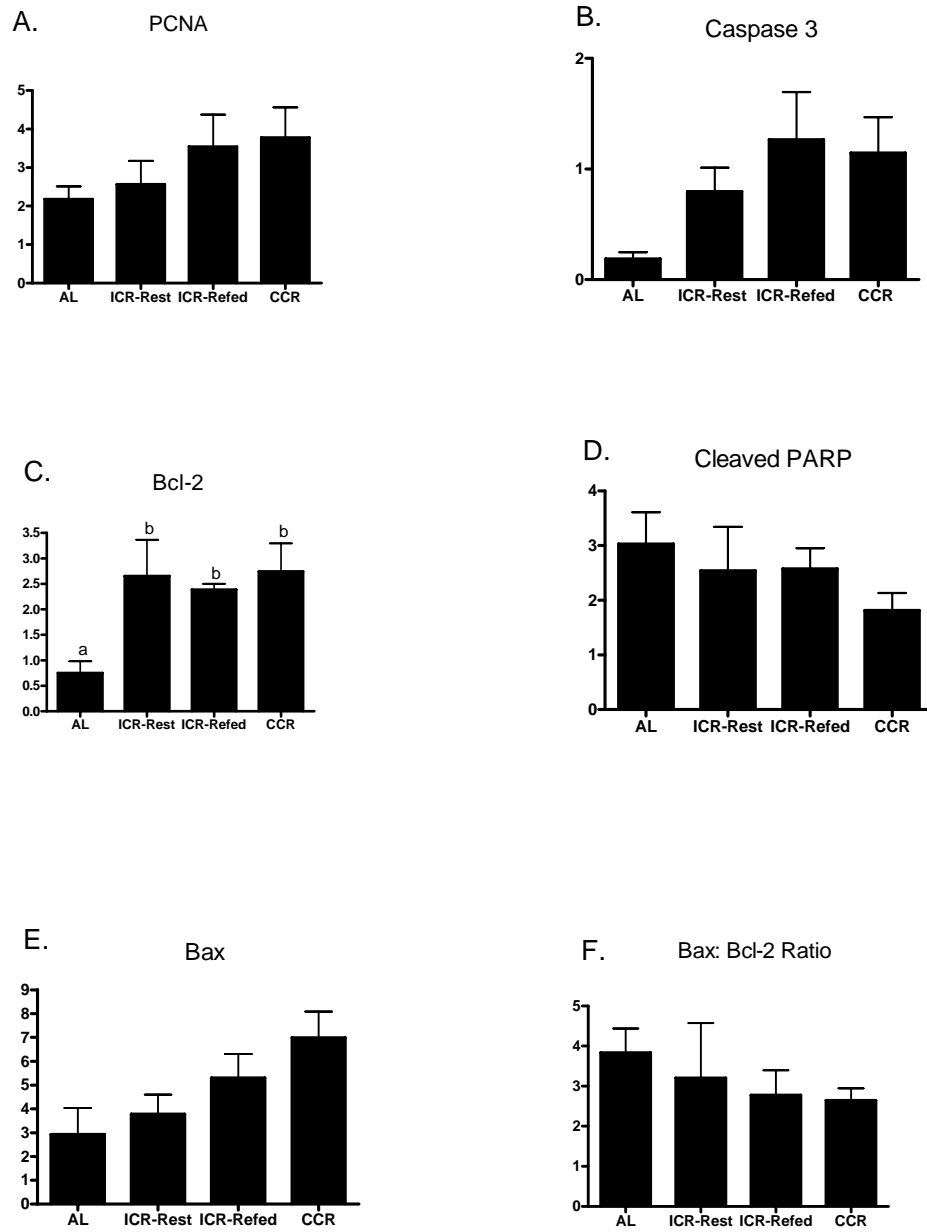
Figure 7. Western blot analysis of tumors tissue in the Longitudinal Study. A. PCNA: AL (N=6), ICR-Rest (N=9), ICR-Refed (N=13), CCR (N=9), P=0.4697, B. Caspase 3: AL (N=4), ICR-Rest (N=8), ICR-Refed (N=8), CCR (N=8), P=0.2322; C. Bcl-2: AL (N=6), ICR-Rest (N=8), ICR-Refed (N=11), CCR (N=9), P=0.0351; D. Cleaved PARP: AL (N=6), ICR-Rest (N=10), ICR-Refed (N=12), CCR (N=9), P=0.5642; E. Bax AL (N=6), ICR-Rest (N=7), ICR-Refed (N=11), CCR (N=9) , P=0.0682; F. Bax: Bcl-2 ratio: AL (N=6), ICR-Rest (N=7), ICR-Refed (N=11), CCR (N=8), P=0.7495.

PROGRESS CROSS-SECTIONAL STUDY

In the Cross-Sectional Study mice were euthanized at predetermined ages for tissue collection. This included 7 (baseline), 16 & 18 (Cycle 3, end of restriction and after two weeks of refeeding), 28 & 30 (Cycle 6, end of restriction and after two weeks of refeeding) and 40 & 42 (Cycle 9, end of restriction and after two weeks of refeeding) weeks of age. The primary goal was to collect tissue samples at various stages of disease development. Summaries of the final body weights and genital-urinary tract weights are shown in Tables 5-7 for ages 16 & 18. 28 & 30, and 40 & 42, respectively. It can be seen that with increasing age, body weight and GU weight increased. In addition more of the mice had tumors detected at euthanasia. With the completion of pathology analyses we have begun analyses of the tissues. We are also planning to use tissue blocks for immunohistochemistry. Proteins of interest included IGF-Receptor, leptin and adiponectin receptors, apoptosis related proteins including Bcl-2, caspase-3 and Bax.

Table 5: Body and Genital-Urinary Tract Weight in TRAMP Mice at 16 and 18 Weeks of Age (mean ± s.e.)

	AL 16 N=9	ICR 16 N=8	CCR 16 N=7
Final BW	27.6 ± 0.67^a	22.4 ± 0.71^b	26.7 ± 0.49^a
GU weight	1.1 ± 0.07^a	0.7 ± 0.07^b	1.0 ± 0.04^a
Tumor detection rate	No tumors	No tumors	No tumors
	AL 18 N=8	ICR 18 N=8	CCR 18 N=8
Final BW	29.1 ± 0.88^a	25.7 ± 0.60^b	28.7 ± 0.64
GU weight	1.1 ± 0.07^a	0.74 ± 0.11^b	1.3 ± 0.11^a
Tumor detection rate	No tumors	No tumors	1 tumor found at dissection

Across a row mice with different superscript letters are significantly different.

Table 6: Body and Genital-Urinary Tract Weight in TRAMP Mice at 28 and 30 Weeks of Age (mean ± s.e.)

	AL 28 N=5	ICR 28 N=7	CCR 28 N=10
Final BW	30.2 ± 0.45	30.4 ± 1.06^a	32.1 ± 0.98^a
GU weight	3.67 ± 0.94^a	1.7 ± 0.15^b	1.8 ± 0.16^b
Tumor detection rate	3	2	3
	AL 30 N=5	ICR 30 N=9	CCR 30 N=6
Final BW	33.7 ± 0.94^a	32.0 ± 1.10^a	31.6 ± 1.27^a
GU weight	3.8 ± 1.25^a	3.2 ± 0.90^a	3.0 ± 1.31^a

| Tumor detection rate	3	5	1

Across a row mice with different superscript letters are significantly different.

Table 7: Body and Genital-Urinary Tract Weight in TRAMP Mice at 40 and 42 Weeks of Age (mean ± s.e.)

|	AL 40 N=3	ICR 40 N=3	CCR 40 N=5
Final BW	34.1 ± 1.73^a	28.3 ± 1.04^a	33.5 ± 1.30^a
GU weight	5.3 ± 0.17^a	2.8 ± 0.29^b	4.3 ± 0.27^c
Tumor detection	3	3	3
	AL 42 N=4	ICR 42 N=4	CCR 42 N=4
Final BW	37.4 ± 1.55^a	30.8 ± 0.95^b	31.1 ± 1.65^b
GU weight	5.4 ± 0.45^a	4.2 ± 0.45^a	3.6 ± 0.45^b
Tumor detection	4	4	3

Across a row mice with different superscript letters are significantly different.

Serum samples were also obtained from the mice at euthanasia in the Cross Sectional study. A summary of the results is presented in Figure 8. At the present time statistical analyses is incomplete. At some data points the n value is small and we are trying to decide how best to analyze the results, i.e., by age or by diet. However, in general it is interesting to note that in line with the terminal serum results serum adiponectin (Figure 8B) levels appear to remain higher with increasing age in the intermittent restricted mice compared to the levels in the other groups.
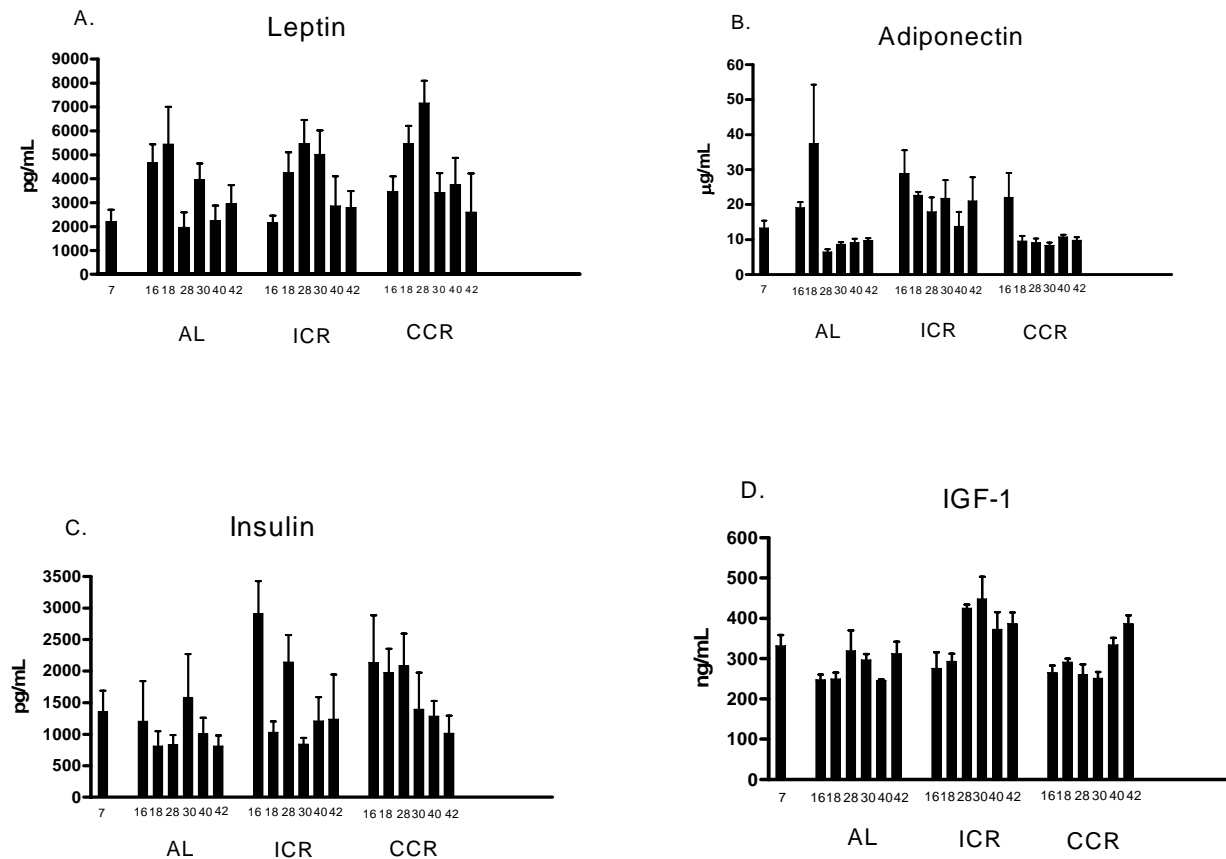
Figure 8. Serum analysis for TRAMP mice in the Cross-Sectional study. A. Leptin B. Adiponectin C. Insulin D. IGF-1. For each graph, TRAMP mice at 7 weeks of age (baseline) are represented by the first bar. Within each dietary group, mice were euthanized at 16, 18, 28, 30, 40 and 42 weeks of age and results are presented in that order.

To date the Western Blot analyses has focused on tumor tissue from mice at 28 and 30 and 40 and 42 weeks of age. No consistent pattern is seen with respect to these proteins and the impact of diet on them as detailed in Figures 9-13.
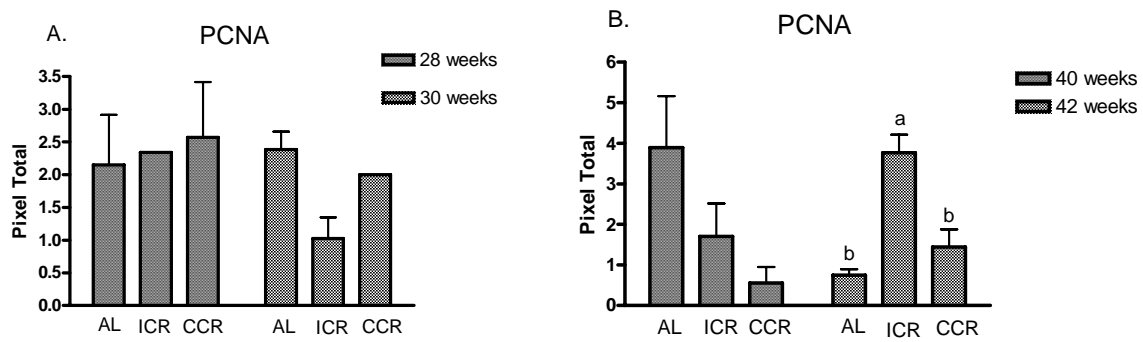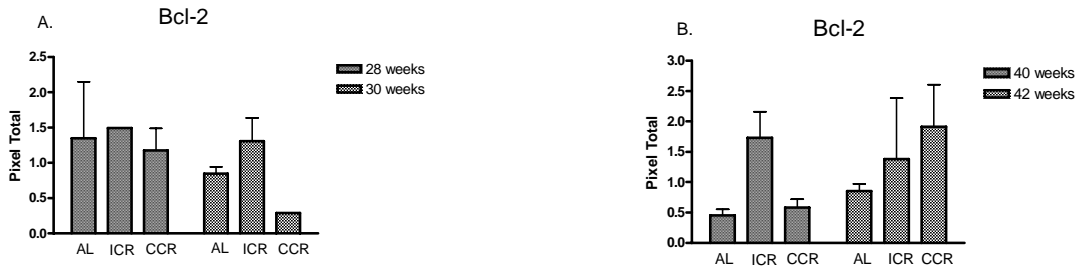
Figure 9. Western blot analysis of tumor tissue in the Cross-Sectional Study. A. PCNA at 28 weeks: AL (N=3), ICR (N=1), CCR (N=3); PCNA at 30 weeks AL (N=3), ICR (N=3), CCR (N=1). B. PCNA at 40 weeks: AL (N=2), ICR (N=3), CCR (N=2), ANOVA P=0.1552; PCNA at 42 weeks: AL (N=3), ICR (N=3), CCR (N=3), ANOVA P=0.0029.
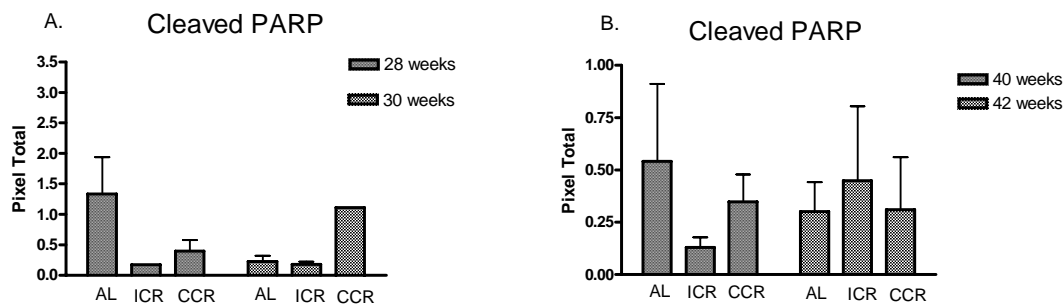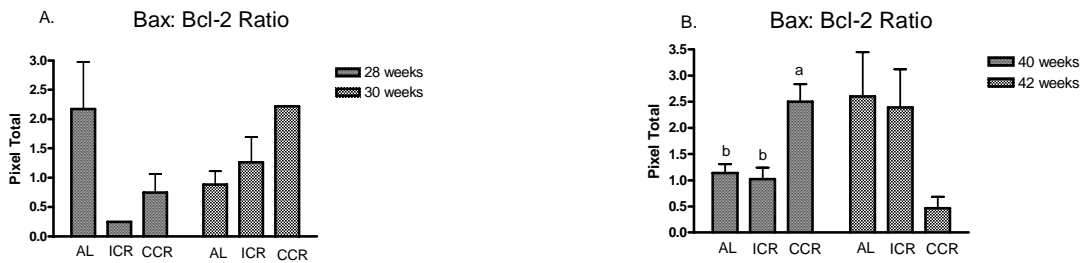

Figure 10. Western blot analysis of tumor tissue in the Cross-Sectional Study. A. Cleaved PARP at 28 weeks: AL (N=3), ICR (N=1), CCR (N=3); Cleaved PARP at 30 weeks: AL (N=3), ICR (N=3), CCR (N=1). B. Cleaved PARP at 40 weeks: AL (N=2), ICR (N=3), CCR (N=3), ANOVA P=0.3429; Cleaved PARP at 42 weeks: AL (N=3), ICR (N=3), CCR (N=3), ANOVA P=0.9068.
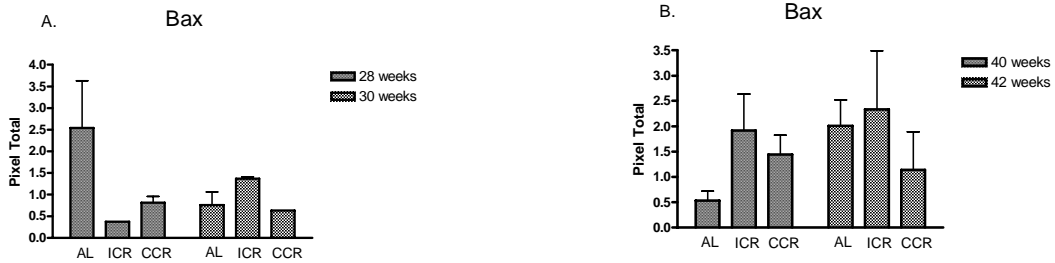
Figure 11. Western blot analysis of tumor tissue in the Cross-Sectional Study. A. Bax at 28 weeks: AL (N=2), ICR (N=1), CCR(N=3); Bax at 30 weeks: AL (N=2), ICR (N=3), CCR (N=1). B. Bax at 40 weeks: AL (N=2), ICR (N=3), CCR (N=3), ANOVA P=0.3188; Bax at 40 weeks: AL (N=3), ICR (N=3), CR (N=3). ANOVA P=0.6170.

Figure 12. Western blot analysis of tumor tissue in the Cross-Sectional Study. A. Bcl-2 at 28 weeks: AL (N=2), ICR (N=1), CCR (N=3); Bcl-2 at 30 weeks: AL (N=3), ICR (N=3), CCR (N=1). B. Bcl-2 at 40 weeks: AL(N=2), ICR (N=3), CCR (N=3), ANOVA P=0.0553; Bcl-2 at 42 weeks: AL (N=3), ICR (N=3), CCR (N=3), ANOVA P=0.5963.

Figure 13. Western blot analysis of tumor tissue in Cross-Sectional Study. A. Bax: Bcl-2 ratio at 28 weeks: AL (N=2), ICR (N=1), CCR (N=3); Bax: Bcl-2 ratio at 30 weeks: AL (N=3), ICR (N=3), CCR (N=1). B. Bax: Bcl-2 ratio at 40 weeks: AL (N=2), ICR (N=3), CCR (N=3), ANOVA P=0.0194; Bax: Bcl-2 ratio at 42 weeks: AL (N=3), ICR (N=3), CCR (N=3), ANOVA P=0.1122.

ORIGINAL-TASK 6. Oncogene and tumor suppressor assays.
MONTHS 6-21. Order supplies and set up assays to perform p53, ErbB2 and possibly other growth factors for determination of gene expression and protein levels Complete setting up assays and analyze samples as they become available. (reviewers indicated not to do this)
REVISED-TASK 6. IGF-BP and IGF-I receptors.

Progress on Task 6. Molecular biology studies of the tissues obtained.
Data are presented above in the context of the Longitudinal and Cross-sectional studies. These proteins remain to be determined but based on other results from mammary tumors we measured a number of proteins associated with apoptosis and proliferation.

TASK 7. Restock breeding colony.

MONTHS 12-14. Evaluate breeding colony status and initiate breeding for
EXPERIMENT 2- FASTING/REFEEDING study.

Progress: This has been completed and we are no longer supporting the breeding colony on this project.

TASK 8. Enroll mice in FASTING/REFEEDING STUDY .
MONTHS 14-21. Breed mice, genotype offspring and enroll mice in FASTING/REFEEDING study. For this study 80-120 mice will be needed depended upon adding a PAIR-FED or a RESTRAINED group. We will have to follow the eating pattern of the FASTING/REFEEDING group for several months to determine if the additional group is needed.

Progress: Due to the longer time frame to enroll the mice in the Intermittent Caloric Restriction studies, mice were not been enrolled in the Fasting/Refeeding study. Also the Hormel Institute instituted Per Diem costs for animal maintenance shortly after this project was funded and it was necessary to pay these costs which has resulted in less money available to carry out these studies. In addition, we did not feel it was prudent to start a new study until we had some idea of what the results of the first study were. Thus due to the much longer time needed for breeding and financial constraints this part of the study was not done.

TASK 9. Serum and tissue analyses and data analyses.
MONTHS 21-24 Complete tissue assays and when all animals are euthanized perform serum analyses and then complete data analyses of EXPERIMENT 1A and 1B.

Progress: IGF-I assays have been completed. We also made arrangements to send the remaining serum samples to University of Pittsburg where Dr. Anna Loshkin

determined insulin, leptin, and adiponectin concentrations. These results were presented above.

TASK 10. Manuscript preparation for EXPERIMENT 1A and B.
MONTHS 25-26 Complete manuscript for the first experiment.

<u>Progress</u>: A draft of the first manuscript describing tumor development is in preparation. Dependent upon results of tissue analyses we hope to have 2-3 additional manuscripts. Three abstracts were presented this past year at AACR meetings. Copies are included in the Appendix.

TASK 11. Follow mice in FASTING/REFEEDING STUDY.
MONTHS 16-30. Monitor food intake, body weight and prostate tumor development in TRAMP mice. When tumor size dictates kill mice and perform autopsies. Kill nontransgenic mice to be age-matched to mice with tumors. Kill mice upon reaching 50 wk of age if still alive.

<u>Progress</u>: Since we did not pursuing this task.

TASK 12. Analysis of tissue samples from FASTING/REFEEDING STUDY.
MONTHS 20-32. Perform assays on tumor and normal tissues from FASTING/REFEEDING STUDY as they become available.

<u>Progress</u>: Since we did not pursue this task there is no progress.

TASK 13. Serum and tissue analyses of FASTING/REFEEDING STUDY.
MONTHS 28-32. Determine serum analyses from FASTING/REFEEDING as study groups are completed. Complete tissue analysis.

<u>Progress</u>: Since we did not pursue this task there is no progress.

TASK 14. Compete statistical analysis of data from FASTING/REFEEDING STUDY.
MONTHS 33-34.Complete statistical analysis of data obtained from the FASTING/REFEEDING STUDY.

<u>Progress</u>: Since we did not pursue this task there is no progress.

TASK 15. Prepare manuscript from FASTING/REFEEDING STUDY.
MONTHS 35-36. Write manuscript from results obtained from FASTING/REFEEDING STUDY.
<u>Progress</u>: Since we did not pursue this task there is no progress.

KEY RESEARCH ACCOMPLISHMENTS:
Our results indicate that intermittent caloric restriction provides greater protection against prostate cancer than does chronic restriction. This is very exciting and confirms previous reports from our laboratory for mammary tumor development. We now plan to

aggressively pursue identifying tissue characteristics and pathways associated with this protective effect and then to apply for funding to continue these studies. Furthermore the leptin to adiponectin ratio data is of interest to study in more detail as to how the interaction of these two serum factors may impact tumor development.

REPORTABLE OUTCOMES

Abstracts were submitted for presentation at the AACR 5th Annual Frontiers in Cancer Prevention Conference November 2006; AACR Innovations in Prostate Cancer Research Meeting December 2006 and for the AACR Centennial Meeting April 2007. Manuscript preparation is underway.

CONCLUSIONS:

Intermittent caloric restriction regimens may provide a useful tool for prostate cancer prevention. Furthermore, identification of the pathways(s) that mediate this protection should allow for rational drug development for cancer prevention. Based on integration of prostate cancer results with those previously obtained in mammary tumor models it appears that this type of intervention may be more effective in slow growing cancers.

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REVISED STATEMENT OF WORK PC020457 2/13/03

Eliminating Specific Aim 5 primarily affects Original Task 6. If there is the opportunity to explore tissue analyses as indicated below it will be focused on aspects of IGF metabolism.

TASK 1 & 2. Establish breeding colony & set up genotyping assay.

MONTH 0-3. Order 6 male TRAMP mice (this is the maximum number that can be ordered at one time) and 25 nontransgenic female mice for breeding. Set up breeding. Set up genotyping assay and genotype mice produced. Rebreed mice to expand breeding colony.

TASK 3. Breed mice for EXPERIMENT 1A- LONGITUDINAL STUDY.

MONTHS 4-6. Breed mice to produce one third to one half of mice needed for this study. If one estimates 8 pups per litter, 1 out of 4 pups will be TRAMP males = two TRAMP males per litter. We will need a total of 160 TRAMP males = 80 litters. Genotype offspring. Assign mice to experimental groups. Set up immunohistochemistry assays.

TASK 4. Complete enrollment of mice for EXPERIMENT 1A and 1B SERIAL STUDY

MONTHS 7-12. Continue breeding to complete EXPERIMENT 1A. Three to four rounds of breeding will probably be needed to supply enough mice. Genotype mice as they are produced. Assign mice to experimental groups. Once longitudinal study is complete begin assigning mice to serial study for EXPERIMENT 1B.

TASK 5. Follow mice in EXPERIMENT 1A and 1B.

MONTHS 6-21. Monitor food intake, body weight and prostate tumor development in TRAMP mice. When age, tumor size and/or animal condition dictates euthanize mice and perform autopsies. Euthanize nontransgenic age-matched mice to correspond to those TRAMP mice with tumors. Euthanize mice that reach terminal ages of 48 or 50 wk of age. Record results and when study complete do statistical analyses of results.

ORIGINAL-TASK 6. Oncogene and tumor suppressor assays.

MONTHS 6-21. Order supplies and set up assays to perform p53, ErbB2 and possibly other growth factors for determination of gene expression and protein levels. Complete setting up assays and analyze samples as they become available.

REVISED-TASK 6. IGF-BP and IGF-I receptors. Since we are focusing on IGF metabolism any work relating to gene expression will concentrate on factors related to IGF-I action such as IGF-I, IGF-I receptors and IGF-BP's.

TASK 7. Restock breeding colony.

MONTHS 12-14. Evaluate breeding colony status and initiate breeding for EXPERIMENT 2- FASTING/REFEEDING study.

TASK 8. Enroll mice in FASTING/REFEEDING STUDY.

MONTHS 14-21. Breed mice, genotype offspring and enroll mice in FASTING/REFEEDING study. For this study 80-120 mice will be needed depended upon adding a PAIR-FED or a RESTRAINED group. We will have to follow the eating pattern of the FASTING/REFEEDING group for several months to determine if the additional group is needed.

TASK 9. Serum and tissue analyses and data analyses.

MONTHS 21-24 Complete tissue assays and when all animals are euthanized perform serum analyses and then complete data analyses of EXPERIMENT 1A and 1B.

TASK 10. Manuscript preparation for EXPERIMENT 1A and B.

MONTHS 25-26 Complete manuscript for the first experiment

TASK 11. Follow mice in FASTING/REFEEDING STUDY.

MONTHS 16-30. Monitor food intake, body weight and prostate tumor development in TRAMP mice. When tumor size dictates kill mice and perform autopsies. Kill nontransgenic mice to be age-matched to mice with tumors. Kill mice upon reaching 50 wk of age if still alive.

TASK 12. Analysis of tissue samples from FASTING/REFEEDING STUDY.

MONTHS 20-32. Perform assays on tumor and normal tissues from FASTING/REFEEDING STUDY as they become available.

TASK 13. Serum and tissue analyses of FASTING/REFEEDING STUDY.

MONTHS 28-32. Determine serum analyses from FASTING/REFEEDING as study groups are completed. Complete tissue analysis.

TASK 14. Compete statistical analysis of data from FASTING/REFEEDING STUDY.

MONTHS 33-34. Complete statistical analysis of data obtained from the FASTING/REFEEDING STUDY.

TASK 15. Prepare manuscript from FASTING/REFEEDING STUDY.

MONTHS 35-36. Write manuscript from results obtained from FASTING/REFEEDING STUDY.

Intermittent caloric restriction delays prostate tumor detection and increases survival time in TRAMP mice

Melissa J.L. Bonorden, Olga P. Rogozina, Michael E. Grossmann, Christina M. Kluczny, Patricia L. Grambsch, Joseph Grande, and Margot P. Cleary

Chronic calorie restriction (CCR) prevents cancer of the breast and prostate in rodent models. For example, in Lobund-Wistar rats restricted by 30% prostate tumors developed at lower rates (Cancer 65:686, 1989), and these rats had increased survival compared to ad libitum fed rats (J Gerontol 45:B52, 1990). Recently, intermittent caloric restriction (ICR) was reported to be more protecive than CCR in two transgenic mouse models that develop mammary tumors (CEBP 11:836, 2002; Nutr Cancer 44:161, 2002), i.e., lower incidences and delayed latency of mammary tumors were detected in ICR mice in comparison to ad libitum fed, as well as to CCR mice. Here, we used the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse to determine how similar caloric restriction protocols, ICR vs CCR, would offer protection against prostate cancer. Male C57BL6 TRAMP mice were assigned to 1) ad libitum (AL, free access to AIN-93M diet), 2) ICR (2-wk of 50% caloric restriction using AIN-93M diet with 2x protein, fat, vitamins, and minerals followed by 2-wk of 100% AL consumption of AIN-93M for each corresponding 2-wk), 3) CCR (fed a diet mixture to match calorie and nutrient intake for each four week ICR cycle ~75% of AL consumption) groups. Protocols were initiated at 7 wk of age and mice followed until disease burden necessitated euthanasia or mice reached terminal endpoints of 48 (last of 11 restrictions) or 50 (last of 11 refeedings) wk of age. Body weights fluctuated in response to calorie intake during the study and were significantly different among the three cohorts ($p < 0.0001$). Final body weights of AL mice were the heaviest followed by ICR-refed, while CCR and ICR-restricted mice weighed the least. Time to tumor detection was significantly different among the three groups (log rank $\chi^2 = 8.736$, 2 degrees of freedom, $p = 0.0127$); occurring at median ages of 33, 35 and 38 wk of age for AL, CCR and ICR mice, respectively. There was no statistical difference for age of prostate tumor detection between AL and CCR mice, while ICR mice were significantly older than both AL ($p = 0.0051$) and CCR ($p = 0.03$) mice. Tumor grade was not different among the groups. A significant difference in survival time was also found among the three groups (log rank $\chi^2 = 12.498$, 2 degrees of freedom, $p = 0.009$). ICR mice had the longest median survival compared to CCR and AL mice, 46, 40 and 41 wk of age, respectively. Forty percent of ICR mice reached their designated terminal end point compared to 27% and 10% for CCR and AL, respectively. Results from this study indicate that mode of caloric restriction impacts both time to tumor detection and survival in TRAMP mice with intermittent restriction providing a greater protective effect compared to chronic restriction. (Support: DAMD17-03-1-0258 and Hormel Foundation)

Comparison of intermittent versus chronic calorie restriction on prostate tumor detection and survival in TRAMP mice

Melissa J.L. Bonorden, Olga P. Rogozina, Michael E. Grossmann, Christina M. Kluczny, Anna Lokshin, Patricia L. Grambsch, Joseph P. Grande, and Margot P. Cleary

Chronic calorie restriction (CCR) prevents prostate tumorigenesis in rodent models. For example, Lobund-Wistar rats calorie restricted by 30% developed prostate tumors at lower rates (Cancer 65:686, 1989) and had increased survival (J Gerontol 45:B52, 1990) compared to ad libitum fed rats. However, intermittent caloric restriction (ICR) was recently reported to be more protecive than CCR in lowering incidence and extending latency in transgenic mouse models that develop mammary tumors (CEBP 11:836, 2002; Nutr Cancer 44:161, 2002) and in reducing metastasis in a lymphoma model (Carcinogenesis 23:817, 2002). Here, transgenic adenocarcinoma of the mouse prostate (TRAMP) mice were used to determine how ICR vs CCR affected prostate cancer development. Male C57BL6 TRAMP mice were assigned to: AL (ad libitum fed AIN-93M diet), ICR (2-wk of 50% caloric restriction of AIN-93M diet with 2x protein, fat, vitamins, and minerals followed by 2-wk of 100% AL mice's consumption of AIN-93M for each corresponding 2-wk), and CCR (fed a diet mixture to match calorie and nutritent intake for each 4-wk ICR cycle, ~75% of AL consumption) groups. Protocols were initiated at 7 wk of age and mice followed until disease burden necessitated euthanasia or mice reached terminal endpoints of 48 (last of 11 restrictions) or 50 (last of 11 refeedings) wk of age. Body weights fluctuated in response to calorie intake during the study (p < 0.0001). Final body weights of AL mice were the heaviest followed by ICR-refed, and CCR and ICR-restricted mice weighed the least. Fat pad weights followed the same pattern. Time to tumor detection was significantly different among the groups (log rank χ^2 = 8.736, 2 degrees of freedom, p = 0.0127); occurring at median ages of 33, 35 and 38 wk of age for AL, CCR and ICR mice, respectively. There was no statistical difference for age of prostate tumor detection between AL and CCR mice, while ICR mice were significantly older than both AL (p = 0.0051) and CCR (p = 0.03) mice. Similar results were found for survival. Tumor grade was not different among the groups. Serum leptin, adiponectin, insulin and IGF-I were all significantly different among the groups (ANOVA p = 0.008, 0.016, 0.003, and 0.007 respectively) Results from this study indicate that the way in which calories are restricted impacts both time to tumor detection and survival in TRAMP mice with intermittent restriction providing a greater protective effect compared to chronic restriction. How this is mediated by growth factors and tissue changes is currently being assessed. (Support: DAMD17-03-1-0258 and Hormel Foundation)

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Delayed prostate tumor detection and increased survival time in TRAMP mice by intermittent calorie restriction

Melissa J.L. Bonorden, Olga P. Rogozina, Michael E. Grossmann, Christina M. Kluczny, Anna Lokshin, Patricia L. Grambsch, Joseph P. Grande, and Margot P. Cleary

A recent study using transgenic female mice indicated that intermittent calorie restriction (ICR) was more protective than chronic calorie restriction (CCR) with respect to lowering mammary tumor incidence and delaying tumor latency (CEBP, 11*: 836, 2002). To examine how these dietary regimes affect prostate cancer development we used both CCR and ICR in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse. Male C57BL6 TRAMP mice were assigned to 1) ad libitum (free access to AIN-93M diet), 2) ICR (2-wk of 50% caloric restriction using AIN-93M diet with 2x protein, fat, vitamins, and minerals followed by 2-wk of 100% AL consumption of AIN-93M for each corresponding 2-wk), 3) CCR (fed a diet mixture to match calorie and nutrient intake for each four week ICR cycle ~75% of AL consumption) groups. Protocols were initiated at 7 wk of age and mice followed until disease burden necessitated euthanasia or mice reached terminal endpoints of 48 (last of 11 restrictions) or 50 (last of 11 refeedings) wk of age. Prior to euthaniasia, terminal body weights and serum were collected.   Final body weights of AL mice were the heaviest followed by ICR-refed, and CCR and ICR-rest weighed the least. GU tract weights and fat pad to carcass ratio followed this pattern. Time to tumor detection was significantly different among the groups (log rank χ^2 = 8.301, 2 = df, p = 0.016); occurring at median ages of 33, 35 and 38 wk of age for AL, CCR and ICR mice, respectively. There was no statistical difference for age of prostate tumor detection between AL and CCR mice, while ICR mice were significantly older than both AL (p = 0.0066) and CCR (p = 0.0416) mice. No differences were found among the three dietary groups with respect to tumor grade (Pearson χ^2 = 2.65, df = 6, p = 0.85). A significant difference in survival time was also found among the three groups (log rank χ^2 = 12.498, df =2, p = 0.009). ICR mice had the longest median survival compared to CCR and AL mice, 46, 40 and 41 wk of age. Forty percent of ICR mice reached their designated terminal end point compared to 27% and 10% for CCR and AL, respectively. Serum leptin, adiponectin, insulin, IGF-1 and leptin to adiponectin ratio were all significantly different among the groups (ANOVA p = 0.0089, 0.0059, 0.0030, 0.0154 and 0.0219). A positive correlation was found between leptin and both fat pad weight (r = 0.5394, p < 0.0001) and final body weight (Peason r = 0.2005, p=0.0066). An inverse correlation was found between adiponectin and final body weight (Pearson r = -0.3249, p < 0.0001).   Western blot analyses of tumor tissue showed no significant differences in expression of caspase-3, PARP, PCNA, bax and bcl-2. Results from this study indicate that ICR increases tumor latency and survival time compared to CCR or AL in TRAMP mice. Further analysis of tissues will hopefully identify mechanisms for this effect. (Support: DAMD17-03-1-0258 and Hormel Foundation).

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Personnel receiving pay from the research effort

Margot P. Cleary

Melissa Bonorden
Michael Grossmann
Christina Kluczny
Nancy Mizuno
Olga Rogozina